

Not entered as it is not in compliance with 37 CFR 1.121; New Matter for complete deletion of the linker sequence (original SEQ ID NO 20) from the entire Specification and Sequence listing and the amendment of the Specification at Table 1 does not correspond to the current Table 1. /vgp/ 6/25/2010

In the Specification:

Please amend the specification as shown:

Please insert the following on page 1, between lines 3-4:

Sequence Listing

The instant application contains a Sequence Listing which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on April 2, 2010, is named ELLIS000.txt and is 10,942 bytes in size.

Please delete the paragraph on page 3, lines 11-24 and replace it with the following paragraph:

The Japanese patents JP02076598 and JP04169195 refer to anti-ED-B antibodies. It is not clear from these documents if monoclonal anti ED-B antibodies are described. Moreover, it seems impossible that a single antibody (such as the antibody described in JP02076598) has "an antigen determinant in aminoacid sequence of formulae (1), (2) or (3):

- (1) EGIPIFEDFVDSSVG **(SEQ ID NO: 22)**
- (2) YTVTGLEPGIDYDIS **(SEQ ID NO: 23)**
- (3) NGGESAPTTLTQQT **(SEQ ID NO: 24)**

on the basis of the following evidence:

- i) A monoclonal antibody should recognise a well-defined epitope.
- ii) The three-dimensional structure of the ED-B domain of fibronectin has been determined by NMR spectroscopy. Segments (1), (2) and (3) lie on opposite faces of the ED-B structure, and cannot be bound simultaneously by one monoclonal antibody.

Please delete the paragraph on page 13, line 27 and replace it with the following paragraph:

Fig. 1 shows a designed antibody phage library. **"VH primers" are disclosed as SEQ ID NOS 11-14, respectively, in order of appearance. "VL primers" are disclosed as SEQ ID NOS 15-18, respectively, in order of appearance;**

Please delete the paragraph on page 19, lines 8-31 and replace it with the following paragraph:

A human antibody library was cloned using VH (DP47; Tomlinson et al. (1992). J. Mol. Biol., 227, 776-798.) and Vk (DPK22; Cox et al. (1994). Eur. J. Immunol., 24, 827-836) germline genes (see Figure 1 for the cloning and amplification strategy). The VH component of the library was created using partially degenerated primers (Figure 1) in a PCR-based method to introduce random mutations at positions 95-98 in CDR3. The VL component of the library was generated in the same manner, by the introduction of random mutations at positions 91, 93, 94 and 96 of CDR3. PCR reactions were performed as described (Marks et al. (1991). J. Mol. Biol., 222, 581-597). VH-VL scFv fragments were constructed by PCR assembly (Figure 1; Clackson et al. (1991). Nature, 352, 624-628), from gel-purified VH and VL segments. 30 µg of purified VH-VL scFv fragments were double digested with 300 units each of NcoI and NotI, then ligated into 15 µg of NotI/NcoI digested pDN332 phagemid vector. pDN332 is a derivative of phagemid pHEN1 (Hoogenboom et al. (1991). Nucl. Acids Res., 19, 4133-4137), in which the sequence between the NotI site and the amber codon preceding the gene III has been replaced by the following sequence (**SEQ ID NO: 25**), coding for the D3SD3-FLAG-His6 tag (**SEQ ID NO: 26**) (Neri et al. (1996). Nature Biotechnology, 14, 385-390):

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      NotI      D  D  D  S  D  D  D
Y   K   D   D
5' - GCG GCC GCA GAT GAC GAT TCC GAC GAT GAC TAC AAG GAC GAC

      D  D  K  H  H  H  H  H  H  amber
GAC GAC AAG CAC CAT CAC CAT CAC CAT TAG - 3'

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Page 14, line 2, please amend as follows:--

Fig. 6 shows amino acid sequence of L19 (VH, **SEQ ID NO: 19** linker and VL, **SEQ ID NOS: 19-21**, respectively).--.

Please delete the paragraphs on page 23, line 1 to page 24, line 14 and replace them with the following paragraphs:

Affinity maturation experiments were performed as follows. The gene of scFv(E1) was PCR amplified with primers LMB1bis (5'-GCG GCC CAG CCG GCC ATG GCC GAG-3' (**SEQ ID**

NO: 1) and DP47CDR1for (5'-GA GCC TGG CGG ACC CAG CTC ATM NNM NNM NNGCTA AAG GTG AAT CCA GAG GCT G-3' (**SEQ ID NO: 2)**) to introduce random mutations at positions 31-33 in the CDR1 of the VH (for numbering: 28), and with primers DP47CDR1back (5'-ATG AGC TGG GTC CGC CAG GCT CC-3' (**SEQ ID NO: 3)**) and DP47CDR2for (5'-GTC TGC GTA GTA TGT GGT ACC MNN ACT ACC MNN AAT MNN TGA GAC CCA CTC CAG CCC CTT-3' (**SEQ ID NO: 4)**) to randomly mutate positions 50,52,54 in CDR2 of the VH. The remaining fragment of the scFv gene, covering the 3'-portion of the VH gene, the peptide linker and the VL gene, was amplified with primers DP47CDR2back (5'-ACA TAC TAC GCA GAC TCC GTG AAG-3' (**SEQ ID NO: 5)**) and JforNot (5'-TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CG-3' (**SEQ ID NO: 6)**) (94C 1 min, 60 C 1 min, 72 C 1 min). The three resulting PCR products were gel purified and assembled by PCR (21) with primers LMB1bis and JforNot (94°C 1 min, 60 C 1 min, 72 C 1 min). The resulting single PCR product was purified from the PCR mix, double digested with NotI/NcoI and ligated into NotI/NcoI digested pDN332 vector. Approximately 9 µg of vector and 3 µg of insert were used in the ligation mix, which was purified by phenolisation and ethanol precipitation, resuspended in 50 µl of sterile water and electroporated in electrocompetent TGI E. coli cells. The resulting affinity maturation library contained 4x10⁸ clones. Antibody-phage particles, produced as described (Nissim et al. (1994). EMBO J., 13, 692-698) were used for a first round of selection on 7B89 coated imunotube (Carnemolla et al. (1996). Int. J. Cancer, 68, 397-405). The selected phages were used for a second round of panning performed with biotinylated ED-B, followed by capture with streptavidin coated magnetic beads (Dynal, Oslo, Norway; see previous paragraph). After selection, approximately 25% of the clones were positive in soluble ELISA (see previous chapter for experimental protocol). From the candidates positive in ELISA, we further identified the one (H10; Table 1) with lowest koff by BIAcore analysis (Jonsson et al. (1991), BioTechniques, 11, 620-627).

The gene of scFv(H10) was PCR amplified with primers LMB1bis and DPKCDR1for (5'-G TTT CTG CTG GTA CCA GGC TAA MNN GCT GCT GCT AAC ACT CTG ACT G (**SEQ ID NO: 7)**) to introduce a random mutation at position 32 in CDR1 of the VL (for numbering: Chothia and Lesk (1987) J.Mol.Biol., 196, 901-917), and with primers DPKCDR1back (5'-TTA GCC TGG TAC CAG CAG AAA CC-5' (**SEQ ID NO: 8)**) and DPKCDR2for (5'-GCC AGT

GGC CCT GCT GGA TGC MNN ATA GAT GAG GAG CCT GGG AGC C-3' (**SEQ ID NO: 9)**) to introduce a random mutation at position 50 in CDR2 of the VL. The remaining portion of the scFv gene was amplified with oligos DPKCDR2back (5'-GCA TCC AGC AGG GCC ACT GGC-3' (**SEQ ID NO: 10)**) and JforNot (94C 1 min, 60 C 1 min, 72 C 1 min) The three resulting products were assembled, digested and cloned into pDN332 as described above for the mutagenesis of the heavy chain. The resulting library was incubated with biotinylated ED-B in 3% BSA for 30 min., followed by capture on a streptavidin-coated microtitre plate (Boehringer Mannheim GmbH, Germany) for 10 minutes. The phages were eluted with a 20 mM DTT solution (1,4-Dithio-DL-threitol, Fluka) and used to infect exponentially growing TG1 cells.

Not entered.

Please delete the header for Table 1 and replace it with the following header:

/vgp/
6/25/2010
column
labels not
present in
amended
Table 1.

Table 1:
Sequences of selected anti-ED-B antibody clones. The column labeled "50-54*" discloses SEQ ID NOS 27, 27, 27, 28 and 28, respectively, in order of appearance. The column labeled "95-98*" discloses SEQ ID NOS 29, 30, 31, 31 and 31, respectively, in order of appearance. The column labeled "91-96*" discloses SEQ ID NOS 32, 33, 34, 34 and 34, respectively, in order of appearance.